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Latex trypsin inhibitors of sweet potato (*Ipomoea batatas* Lam.)

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Abstract. Levels and electrophoretic patterns of trypsin inhibitors (TIs) and sweet potato (*Ipomoea batatas* Lam. cv. Tainong 57) latex proteins were analyzed. Levels were determined by spectrophotometry. Electrophoretic patterns of SDS-polyacrylamide gels include protein, TI activity (TIA), and peroxidase-anti-TI stainings. The following results were obtained: **1)** Total TIA: 54.97 ± 7.929 mg (n = 6 roots) tryspin inhibited per 100 g latex; specific TIA: 0.0512 ± 0.01350 mg (n = 6) trypsin inhibited per mg protein. **2)** Sweet potato latex contained four major polypeptides (M_r 72400, 47800, 40900, and 20100), one major TIA band (M_r 20100), four minor TIA bands (M_r 72400, M_r 47800, M_r 40900, and M_r 34600), and three TI or TI-related antigens (M_r 72400, M_r 47800, and M_r 34600). **3)** TI and TI-related polypeptides are major polypeptides of latex. The possible systemic functions played by latex TIs is discussed.

Keywords: Activity; Activity staining; Electrophoresis; Peroxidase-anti-TI staining.

Abbreviations: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; **SP,** sweet potato; **'T57'**, sweet potato variety Tainong 57; **TIA**, trypsin inhibitor activity.

Introduction

The occurrence of proteinase inhibitors in plant storage organs such as seeds, tubers, and storage roots is widespread. They have been isolated primarily from the Leguminosae, Graminae, and Solanaceae, and from a number of other families (Ryan, 1973).

Trypsin inhibitor activities (TIA) are positively correlated with protein concentration in dormant roots of 53 sweet potato (SP) varieties grown in the field under similar conditions, suggesting that TI is a root storage protein (Lin and Chen, 1980). The relationship was confirmed by Bouwkamp et al. (1985) for 12 SP clones grown in the field for four seasons. Maeshima et al. (1985) identified sporamins as major proteins in tuberous roots of one SP cultivar, with no other functions detected. Bradshaw et al. (1989) demonstrated that systemically wound-responsive genes in poplar trees encode proteins similar to SP sporamins and legume Kunitz TIs. Independently, we found that rabbit antiserum against SPTI with a relative mobility 0.451 (M_r 34800) or against SPTI with a relative mobility 0.672 (M_r 33100) crossreacted with other SPTIs and soybean Kunitz TI (Lin, 1990). Polygalacturonic acid is a potent inducer of proteinase inhibitor genes in plants. It is probably released by the hydrolysis of cell wall polysaccharides upon wounding, and mediates the induction of wound response (Ryan and Farmer, 1991). Recently, the induction of expression of genes coding for sporamin and β-amylase by polygalacturonic acid in leaf-petiole cuttings of SP was reported (Ohto et al., 1992). We have accumulated lines of evidence suggesting that sporamins are actually TIs, or at least are strongly related to TIs (unpublished data).

Latex occurs in 12500 species belonging to 900 genera (Esau, 1965). Metcalfe's records (1983) show that the plants concerned belong to more than twenty-two families, mostly dicotyledons but a few monocotyledonous families are included. Latex is typically contained in tubes and cells which are collectively known as laticifers. Laticifers have been recorded in the cortex and phloem of *Ipomoea* (Metcalfe, 1950). We have detected, for the first time in literature, positive TI activity staining in latex and in regions around the cortex and phloem (Lin, 1990).

The objectives of this study were: **1)** to compare levels of latex TIA and water-soluble proteins with those of other SP tissues, **2)** to investigate electrophoretic patterns of latex crude extract on SDS-PAGE gels, including protein, TIA, and peroxidase-antiTI stainings of SP Tainong 57 ('T57').

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Materials and Methods

Plant material

Sweet potatoes (*Ipomoea batatas* Lam. cv. Tainong 57) were purchased from a local market. Roots were washed with water, sprayed with 0.04% sodium azide, and allowed to sprout for three weeks in darkness, programmed at 30 ± 1 °C (12h) and 27 ± 1 °C (12h) with 70 ± 2% relative humidity. Sprouts, weighing between 8.39 and 17.62 g, were rinsed with water, sprayed with 70% ethanol, washed three times with distilled water, and wiped dry.

For comparison, some roots with sprouts were rinsed with water, sprayed with 70% ethanol, washed three times with distilled water, and divided into four parts: **1**) sprouts, **2**) one cubic centimeter of root area where sprouts originated, **3**) the half root area where sprouts were removed minus part 2, and **4**) the half root area where no sprouts were produced. Some of the sprouting sweet potatoes were moved to a growth chamber (16 h light at 22 °C, 290μE m^{-2} s^{-1} photon flux density; 8 h dark at 17 °C; and a relative humidity of 85%) for another two weeks to obtain stems, young leaves, and old leaves. Materials were sterilized and used directly or frozen at -17 °C.

Mungbean (*Vigna radiata* L. cv. Tainan 3), pea (*Phaseolus vulgaris* L. cv. Blue Lake) were also purchased from the local market and treated similarly.

Chemicals

Acrylamide, ammonium persulphate, and N, N'-methylene bisacrylamide were products of Bio-Rad (Richmond, CA, USA). Calibration kit for electrophoresis was obtained from Pharmacia (Uppsala, Sweden). It included bovine milk α-lactalbumin (M_r 14400); soybean trypsin inhibitor (M_r 20100); bovine erythrocyte carbonic anhydrase II (M_r 30000); chicken egg white ovalbumin (M_r 43000); bovine serum albumin (M_r 67000); and rabbit muscle phosphorylase b (M_r 94000). L-ascorbic acid, polyvinylpyrrolidone-40 (M_r 40000), soybean Kunitz-type TI (T-9128, type II-S), limabean (*Phaseolus limensis* Macfady) TI (T-9378, type II-L), chicken egg white TI (T-2011, type III-O), bovine pancreas TI (T-0256, type I-P), and other chemicals were obtained from the Sigma Chemical Company (St. Louis, MO, USA).

Crude extracts

The sprouts of each root provided 0.04 g of latex and were handled separately. A total of 6 roots were used in a series of experiment. The following procedures were carried out at 4 °C. Latex was squeezed from washed sprouts into 0.2 ml of 10 mM phosphate buffer (pH 7.0) containing 1% L-ascorbic acid and then homogenized. The homogenate was centrifuged at 177000 x *g* for 15 min. The supernatant liquid was collected as crude extract and used immediately after preparation.

For crude extracts of other plant tissues, the following procedures were also carried out at 4 °C. Samples were cut into small pieces in five volumes of 10 mM phosphate buffer (1 g sample:5 ml buffer) containing 1% polyvinylpyrrolidone and 1% ascorbic acid with a final pH of 7.0, and then homogenized in a Polytron (Luzern, Switzerland) homogenizer. The homogenate was centrifuged at 177000 x *g* for 60 min, the supernatant liquid was collected, glycerol and NaN_3 were added to a final concentration of 25% and 0.1%, respectively, and then it was stored at -20 °C until the assays.

Protein estimation

Protein was determined with the Folin phenol reagent (Lowry et al., 1951) using crystalline bovine serum albumin as the standard.

Determination of trypsin inhibitor activity

Assay of TIA followed Lin and Chen's (1980) modification of Kunitz's method (Kunitz, 1964).

SDS-polyacrylamide gel Electrophoresis

SDS-PAGE was done according to Weber and Osborn (1969). Gradient gels of 10–20% or gels of 15% were used. Protein was detected with Coomassie Brilliant Blue (Neuhoff et al., 1988) after electrophoresis.

Post-electrophoresis TIA staining

This was carried out according to Chan and deLumex (1982). For SDS-PAGE, SDS must be removed before TIA staining (Lin, 1990). Gels were incubated in 0.1 M phosphate buffer (pH 7.4) containing 0.05 mg/ml trypsin for 20 min at 37 °C while swirling the solution in a covered glass tray about every 5 min. The gel was rinsed with 25 to 50 ml of water and allowed to sit for 10 min at 37 °C in a covered tray. The gel was then incubated with 160 ml of substrate-dye solution (prepared immediately before use), which consisted of 40 mg of N-acetyl-DL-phenylalanine-β-naphthyl ester in 16 ml of dimethylformamide brought to 160 ml with 144 ml of 0.05 M phosphate buffer (pH 7.4) in which 80 mg of tetrazotized *o*-dianisidine were dissolved. Gels were destained with 2% acetic acid for 30 min and stored in methanol: acetic acid: water (5:1:5) for 30 min. The gel was then destained in the same solvent. Clear bands were obtained for TIA and dark bands were obtained for plant proteases.

Post-electrophoresis enzyme-immuno-blotting of trypsin inhibitor

Polyclonal antibody against TI of SP roots (M_r 34600) was raised from rabbit (Lin, 1990). Western blotting of proteins on hydrophobic polyvinylidene difluoride-based membranes (Immobilon) was as described by Matsudaira (1987). Horseradish peroxidase-immuno-staining was performed according to the Immobilon Tech Protocol provided by Millipore (1990).

Table 1. Levels of water-soluble protein and trypsin inhibitor activity of latex of sweet potato 'T57'.

Root	Root weight (g)	sprout weight (g)	Latex weight (g)	Total[a] TIA (mg)	WS[a, b] protein	Specific[c] TIA
1	598.53	17.62	0.04	51.63 ± 0.408[d]	1425 ± 10.0	0.0362 ± 0.00290
2	447.49	12.15	0.04	66.10 ± 4.103	1205 ± 12.5	0.0549 ± 0.00341
3	409.92	8.56	0.04	53.58 ± 0.513	790 ± 10.0	0.0678 ± 0.00651
4	314.60	13.30	0.04	40.55 ± 3.183	1145 ± 5.0	0.0353 ± 0.00277
5	286.36	8.39	0.04	58.65 ± 2.158	930 ± 4.5	0.0631 ± 0.00232
6	227.35	8.44	0.04	59.30 ± 5.728	1195 ± 5.5	0.0497 ± 0.00480

[a]Data were determined and calculated on the basis of 100 g latex. TIA, trypsin inhibitor activity. One unit of TIA inhibits 1mg trypsin.
[b]WS, water-soluble.
[c]TIA, units of TIA per mg protein. Obtained by dividing total TIA by WS protein.
[d]Mean ± standard error (n = 3).

Table 2. Levels of total water-soluble protein and trypsin inhibitor activity in various tissues of sweet potato 'T57'[a].

	Latex	Stem	Leaves		Sprouted root			
			Young[b]	Old[c]	Sprouts without leaves	Part 2[d]	Half root area where sprouts were removed, minus part 2	Half root area where no sprouts originated
Total TIA[e]	55.0	18.7	384.7	220.7	394.2	439.6	303.0	232.4
Total WS[f] proteins (g)	1.12	0.687	1.88	1.97	1.91	1.62	1.53	1.96
Specific TIA[g]	0.0512	0.027	0.205	0.112	0.206	0.272	0.199	0.119

[a] Data were determined and calculated on the basis of 100 g fresh weight.
[b] Young leaves, light green color, with length less than 3 cm and width less than 3.6 cm.
[c] Old leaves, dark green color, with length between 5 and 7 cm, width between 5.6 and 8.2 cm.
[d] One cubic centimeter of root area where sprouts originated.
[e] TIA, trypsin inhibitor activity. One unit of TIA inhibits 1 mg trypsin.
[f] WS, water-soluble.
[g] Specific TIA, units of TIA per mg protein. Obtained by dividing total TIA by total WS protein.

Sequence comparison

Sequence comparisons between bovine carbonic anhydrase II and soybean TI, and between bovine milk α-lactalbumin and soybean TI were carried out by a computer software program called GAP, in the Sequence Analysis Software package, version 7, provided by Genetics Computer Group, Inc. (1992). GAP makes an alignment to find the maximum similarity between two sequences by the method of Needleman and Wunsch (1970). Values of 3.000 and 0.100 were set for gap weight and length weight, respectively.

Results

Levels of trypsin inhibitor and water-soluble protein in 'T57' latex

Table1 shows levels of TIA and water-soluble protein of latex in 6 'T57' roots. In general, they are very similar. They average about 1.12% protein (fresh weight) and all have significant TI levels. Because latex coagulates rapidly after being squeezed from tissues, and because the amount of latex is small, about the same amount (0.04 g in this experiment) of latex was obtained from each root in spite of almost a 2:1 variation (17.62 to 8.39 g) in the weight of sprouts from roots 1–6.

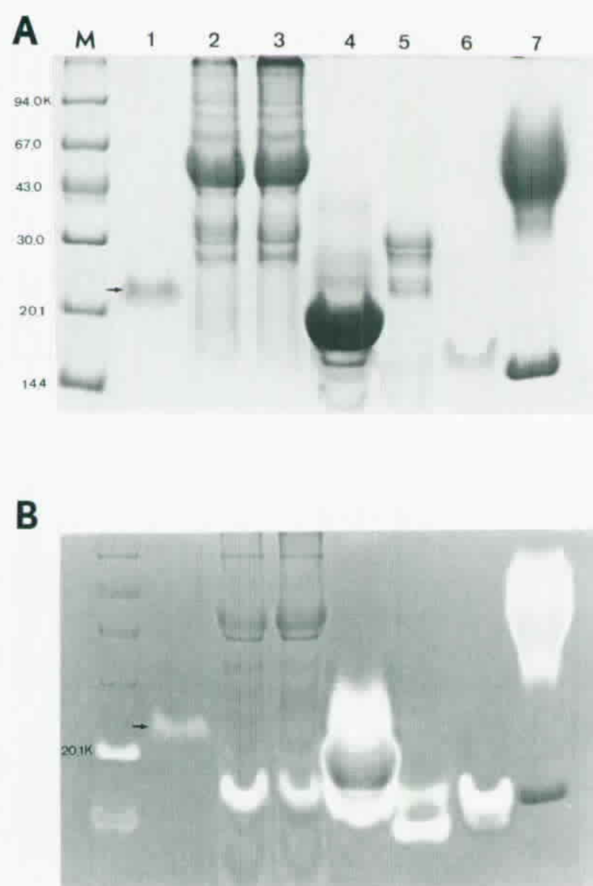

Figure 1. Specificity of polyclonal antibody against TI of sweet potato as revealed by enzyme-immuno-blotting on 15% SDS-polyacrylamide gels. A, protein staining; B, TI-activity staining. Lane M, low molecular weight kit; lane 1, SPTI M_r 25500; lane 2, crude extract of mungbean; lane 3, crude extract of pea; lane 4, soybean Kunitz-type TI; lane 5, limabean TI; lane 6, bovine pancreas TI; lane 7, chicken egg white TI. E. ach lane, excepting lanes M and 1, contains 40 μg protein; lane M contains 43.95 μg protein and lane 1 contains 20 μg protein. The anode runs from top to bottom.

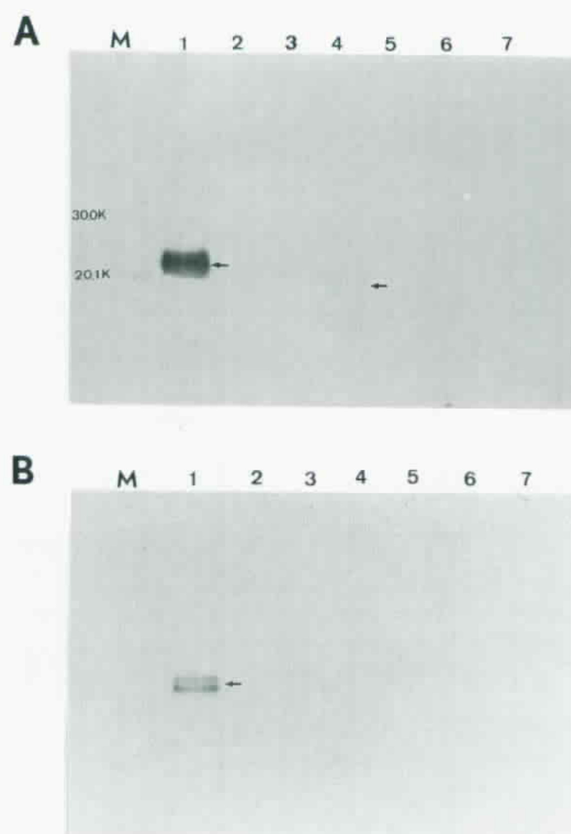

Figure 2. Specificity of polyclonal antibody against TI of sweet potato as revealed by enzyme-immuno-blotting on 15% SDS-polyacrylamide gels. A and B are peroxidase-anti-TI34600 blotting patterns. Lane M, low molecular weight kit; lane 1, SPTI M_r 25500; lane 2, crude extract of mungbean; lane 3, crude extract of pea; lane 4, soybean Kunitz-type TI; lane 5, limabean TI; lane 6, bovine pancreas TI; lane 7, chicken egg white TI. E. ach lane, excepting lanes M and 1, contains 2 μg in A, and 10 μg in B; lane M contains 17.58 μg protein in A, and 35.16 μg in B. Lane 1 contains 2 μg protein in A, and 10 μg in B. The anode runs from top to bottom.

Table 2 shows that levels of total TIA, total water-soluble protein, and specific TIA in latex were lower than that in all other sweet potato tissues except stem, on a fresh weight basis.

Specificity of polyclonal antibody against SPTI revealed by peroxidase-immuno-staining

Figures 1A, 1B, 2A, and 2B show that our polyclonal antibody against SPTI (M_r 34600) is quite specific and crossreacts weakly with soybean Kunitz-type TI, crude extracts of both mungbean and pea, and bovine carbonic anhydrase included in Pharmacia low molecular weight kit. Crossreaction of our antibody against SPTI to bovine carbonic anhydrase was unexpected, but is reasonable and will be discussed later. In addition, bovine milk α-lacalbumin revealed a weak positive TIA staining. This was also unexpected, but it is possible as will be discussed later.

Electrophoresis patterns of crude extracts of 'T57' latex

Figure 3A shows electrophoretic protein-staining patterns of latex crude extracts of 6 'T57' roots. SP latex contains four major polypeptides, with M_r 72400, 47800, 40900, and 20100. Figure 3B shows that SP latex contains one major TIA band (M_r 20100), four minor TIA bands (M_r 72400, M_r 47800, M_r 40900, and M_r 34600), and a broad TIA band with M_r less than 20100. Three TI or TI-related antigens (M_r 72400, M_r 47800, and M_r 34600) were observed in SP latex (Figure 3C). In summary, TI and TI-related polypeptides are major polypeptides of SP latex.

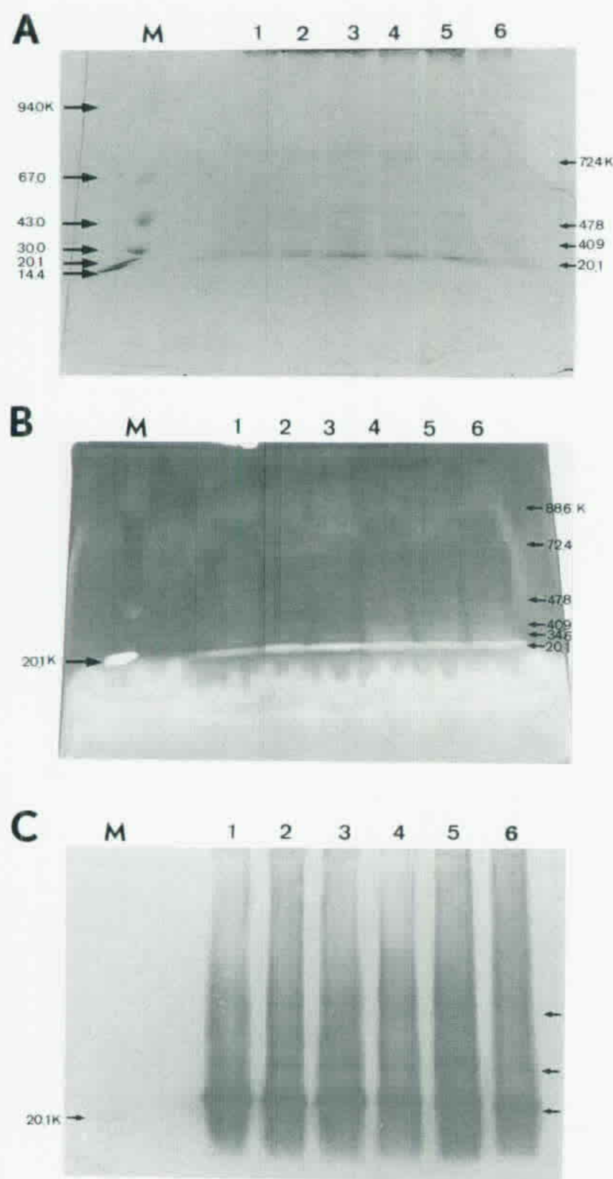

Figure 3. Electrophoretic patterns of crude latex extract of SP 'T57' on 10–20% gradient SDS-polyacrylamide gels. Lane M is the low molecular weight kit. Lanes 1–6 contain samples from roots 1–6, respectively. The anode runs from top to bottom. **A**) Protein staining; **B**) TI activity staining; **C**) peroxidase-anti-TI34600 blotting. Peroxidase-anti-SPTI staining was performed after electrophoresis and blotting. Lane M contains 10 μg protein each in A and B, 2.5 μg protein in C. Lanes 1–6 contain 35 μg protein each in A and B, 10 μg protein in C.

Discussion

Although levels of total TIA and total water-soluble protein of latex are lower than in all other SP tissues, except stem, on a fresh weight basis (Table 2), these will become much higher if comparison is based on dry weight basis because latex is a liquid. The consistency in the order of listings of both total TIA and specific TIA in eight tissues of 'T57' (Table 2) reflects the importance of TIs among storage proteins in this cultivar (Lin and Chen, 1980; Lin, 1988 and 1989).

Our polyclonal antibody against SPTI is quite specific. Crossreaction of our antibody with bovine carbonic anhydrase II was somewhat unexpected, hence we compared the sequences between bovine carbonic anhydrase II (total amino acids: 259) and soybean Kunitz-type TI (total amino acids: 216) with the GAP program. Surprisingly, both proteins have 43.37% similarity and 19.28% identity. Therefore, crossreaction of our antibody with bovine carbonic anhydrase II is reasonable and provides additional evidence for the specificity of our antibody. Bovine milk α-lacalbumin revealed a positive TI-activity staining (Fig. 1B). This was also unexpected, but is possible because bovine milk α-lacalbumin (total amino acids: 142) shares 44.78% similarity and 17.16% identity with SP sporamin A precursor, and 49.25% similarity and 17.91% identity with soybean Kunitz type TI. One thing in common between bovine milk and sweet potato latex is that both are secretory body fluids. Although there is a significant difference between the structure and physiology of secretory organs in animals and plants, the occurrence of macromolecules with TIA in bovine milk and SP latex is probably not mere chance. Since latex occurs in 12500 species (Esau, 1965) and laticifers are specialized cells or tubes of many species including *Ipomoea batatas* (Metcalfe, 1950), TIs in latex may play some important systemic functions.

Figure 3 shows that TIs are major soluble polypeptides of SP latex in roots, sprouts, and stems (not shown). The activity bands with M_r less than 20100 (Figure 3B) were also observed in stems and leaves (Lin, 1993) and sprouts (unpublished data). As in other experiments, the broad TIA band was not detected by our polyclonal antibody against TI34600 (Figure 3C) (data not shown). This observation suggests that the region(s) recognized by anti-TI34600 may be different from active site(s) of TIs. The identity of TIs smaller than 20100, especially those smaller than 18000, needs further study. There are two new activity bands, M_r 72400 and M_r 47800, while activity band M_r 88600 is less reliable and needs further confirmation.

Although SPTIs with M_r 40900 and M_r 34600 revealed only weak TIA activity (Figure 3B), both were identified positively in enriched partially purified samples of 'T57' roots (Lin, 1990). Our antibody against SPTI with M_r 34600 detected a major TI band with M_r 34600, a minor TI with M_r 47800, and a TI-related protein with M_r 72400 (Figure 3C).

Watson et al. (1992) mentioned that all the proteins that have been sequenced so far are made up of only some 1000 to 7000 different exons. This principle of thrift can also be observed in sweet potato by the existence of TI-related polypeptides, namely the one with M_r 72400 (Figure 3C) and the second one with M_r 55000,

which was detected in other tissues of SP by our antibody against SPTI with M_r 34600 (unpublished data). The latter is exactly of the same M_r as the subunit of SP β-amylase. The GAP program shows that SP β-amylase shares 45.12% similarity and 17.67% identity with sporamin A precursor; this may explain why our antibody against SPTI cross-reacted with the polypeptide with M_r 55000.

Bradshaw et al. (1989) demonstrated that systemically wound-responsive genes in poplar trees encode proteins similar to SP sporamins and legume Kunitz TIs. Independently, we found that rabbit antiserum against SPTI with a relative mobility of 0.451 (M_r 34800) or SPTI with a relative mobility of 0.672 (M_r 33100) crossreacted with all other SPTIs and with soybean Kunitz TI (Lin, 1990). We also found that 8 out of 10 N-terminal amino acids of a TI (M_r 18000), which is one of two subunits of a TI with a relative mobility of 0.398 (M_r 39000), are the same as that reported for sporamin A (Maeshima et al., 1985).

As far as TIs with M_r 18000–39000 are concerned, all data suggest that sporamins are TIs, or at least highly related to TIs. The identity of TIs with M_r smaller than 18000 or larger than 39000 remains to be elucidated.

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甘藷乳汁的胰蛋白酵素抑制因子

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本實驗分析台農 57 號甘藷 (*Ipomoea batatas* Lam.) 乳汁粗抽液蛋白質含量和胰蛋白酵素抑制因子（以下簡稱 TI）活性，及其硫酸十二酯鈉-聚丙烯醯胺電泳之花樣。TI 活性以吸光法測定。電泳後分別染蛋白質， TI 活性，及 TI 抗原。結果綜述如下：1. 總 TI 活性爲 100克乳汁可抑制 54.97±7.929 (n = 6 個塊根) 毫克胰蛋白酵素；而 TI 比活性爲每一毫克之乳汁蛋白質可抑制 0.0512±0.01350 (n = 6) 毫克胰蛋白酵素。2. 乳汁含四種主要多肽分子量 (M_r) 分別爲72400，47800，40900，及 20100；一種主要 TI 活性帶 (M_r爲 20100) 和四種次要 TI 活性帶 (M_r 分別爲 72400，47800，40900，及 34600)；三種 TI 或 TI-相關之抗原 (M_r 分別爲 72400，47800，及 34600)。3. TI 或 TI-相關之多肽爲乳汁的主要多肽。乳汁的 TI 可能和甘藷某些全身之功能有關。

關鍵詞: 活性；電泳；活性染；過氧化酵素-TI 抗體染色。